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Toxicity Test Using Life Stages of 'Champia Parvula' (Rhodophta)

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A Toxicity Test Using Life Stages of *Champia parvula* (Rhodophyta)

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KEY WORDS: macroalgal toxicity test, aquatic toxicology, silver, copper, cadmium, lead, cyanide, growth inhibition, reproduction, algae, hazard assessment

New and exotic chemicals, as well as many known compounds are being released into the air and water daily. Most of these compounds find their way into the marine environment and need to be tested as potential toxicants to different trophic levels of marine organisms. Standardized toxicological testing procedures have been developed for fish, invertebrates, amphibians, and microalgae [ASTM Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians (E 729-80)] [*1,2*].[2] How-

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[2]The italic numbers in brackets refer to the list of references appended to this paper.

ever, very little work has been done toward the development of a standard procedure for testing the effects of toxicants on macroalgae. Macroalgae have been used to monitor pollution of heavy metals in the field [*3,4*]. This type of study requires the use of relatively tolerant species since the goal is to have an alga that will accumulate a pollutant without dying [*5*]. In toxicological studies, the opposite is true. One looks for a relatively sensitive organism, because if sensitive organisms are protected, tolerant species should be protected as well. *Champia parvula* (C. Ag.) Harv. is a common species in many parts of the world [*6–9*] and may be a useful surrogate species for determining the relative toxicity of a variety of chemicals.

The primary objectives of this study were to develop a static-renewal exposure system, to evaluate the utility of various end points, and to determine the relative sensitivity of these end points. Test results encompass vegetative growth, sexual reproduction, and formation of tetrasporangia. *Champia parvula*, a marine red alga, was chosen because of its ease of maintenance in culture, its short life cycle, and its relative sensitivity to several of the toxicants tested.

Materials and Methods

As do many red algae, *C. parvula* has three free-living isomorphic plants in its life history: haploid male and female gametophytes and a diploid tetrasporophyte (Fig. 1). In culture the entire life cycle takes approximately six weeks to complete. All three plants of the life history can be maintained in separate unialgal cultures. New cultures can be started from excised branches, making it possible to maintain clonal material indefinitely. No special preconditioning is required to induce reproduction. Under the culture conditions listed in the following sections, male gametophytes produce spermatia continuously, female gametophytes are always receptive, and tetrasporophytes constantly produce tetrasporangia. Thus, plant material can be available at any time for experiments.

Maintenance of Stock Cultures

Unialgal stock cultures were maintained in screw-capped 3.8-L flint-glass jars containing 3 L of a one-fourth strength modified medium f [*10*]. The diluted medium consisted of 37.5 mg sodium nitrate ($NaNO_3$), 2.5 mg sodium phosphate ($NaH_2PO_4 \cdot H_2O$), 0.33 mg iron [as ferric chloride ($FeCl_3$)], and 2.18 mg disodium salt of ethylenediaminetetraacetic acid (EDTA) per litre of filtered seawater (15-μm charcoal filter and 0.3-μm Balston[3] filter). No vitamins, silica, or trace metals were added to the cultures. Seawater was

[3]Mention of trade names does not imply the endorsement of the U.S. Environmental Protection Agency.

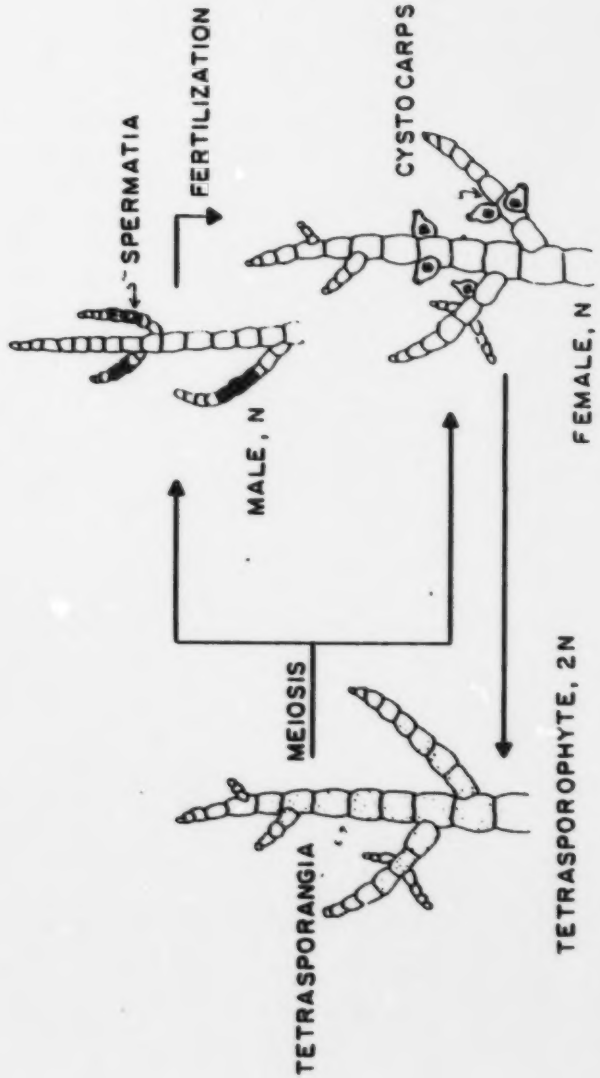

FIG. 1—*Life history of the marine red alga* Champia parvula *(C. Ag.) Harv.*

autoclaved in 20-L linear polyethylene carboys for 25 min at 1.1 kg/cm² (15 psi) after the salinity was adjusted with deionized water (if necessary) to 30 parts per thousand. The flint-glass culture jars were sterilized without breakage by putting about 2 L of deionized water into each jar and autoclaving them partially submerged in water (about 5 cm deep). The media were prepared by pouring out the deionized water, filling the jars with 3 L of sterile seawater, and adding 5 mL of a sterile nutrient stock solution to give the previously mentioned nutrient concentrations. The media were changed once a week. The jars were capped with autoclavable polypropylene lids (no paper liner). A ~5-mm diameter hole was drilled for insertion of an air tube, and the hole was stoppered with a foam Identi-plug.[3] The cultures were gently aerated with aquarium pumps through disposable polystyrene, 1-mL pipettes. The cultures were illuminated from the side at a light intensity of ~75 μeinsteins/m²·s of cool white fluorescent light on a 16:8, light:dark cycle. The temperature was 20 to 22°C.

The initial maintenance cultures were started weekly with approximately twenty 0.5- to 1.0-cm branch tips. To reduce the amount of biomass as the plants grew, about half of the plants were discarded with each weekly medium change. At the end of three weeks the plants were ready to be used in toxicity tests. With this procedure, actively growing plants were continuously available.

Test Procedure

The toxicity tests lasted 11 days for the tetrasporophytes and 14 days for the females. The tests were performed with 400 mL of medium in screw-capped or silicone-rubber-stoppered 500-mL Erlenmeyer flasks. Before use, the flasks were washed, acid stripped overnight with 10% hydrochloric acid (HCl), rinsed with 18 mΩ deionized water, and autoclaved at 1.1 kg/cm² (15 psi) for 10 min. All of the medium was mixed in a single batch. The medium for the toxicity tests consisted of 15 mg/L $NaNO_3$, 1 mg/L $NaH_2PO_4 \cdot H_2O$, 15 μg/L iron, and 100 μg/L EDTA. In addition, 150 mg/L sodium bicarbonate ($NaHCO_3$) was added. The medium was altered from that for maintenance cultures because the toxicity test cultures had a lower biomass-to-volume ratio than the maintenance cultures. The EDTA was reduced to minimize interference with heavy metal toxicity. *Champia parvula* would not grow well in the complete absence of EDTA. The bicarbonate was added because it stimulated the growth of *C. parvula* (presumably, it acted as a carbon source since the flasks were sealed). The media were replaced on Day 7 for the tetrasporophytes and on Days 7 and 11 for the females.

After the plants were rinsed in sterile seawater, to remove traces of old media, 2- to 3-mm branch tips were cut to serve as innocula for a toxicity test. The tips were cut with stainless steel, fine-point forceps. One three-week-old plant supplied all the tips needed for a given test. Five tips of the life cycle stage in question were placed into each test flask. One male branch (about 1 cm long), visibly producing spermatia, was added to each flask containing females. When the media were changed, the male branches were clipped back to 1 cm to minimize their competition for nutrients with the female plants. All the tests were performed in duplicate. The flasks were illuminated from above at ~75 μeinsteins/m²·s with cool white fluorescent light. The photoperiod was a 16:8 light:dark cycle. The temperature was 20 to 22°C, and the salinity was 30 parts per thousand. The flasks were shaken on a rotary shaker at 100 rpm. Although *C. parvula* grew better with aeration than with shaking, the latter was selected as a standard method to minimize the loss of volatile toxicants.

End points

The end points examined at the termination of a toxicity test were the number of tetrasporangia per milligram/dry weight the presence or absence of cystocarps, and the vegetative growth of tetrasporophytes and females (as measured by final dry weight). The tetrasporangia were counted by placing the plants between the inverted halves of a polystyrene petri dish with a small volume of seawater (to hold the entire plant in one focal plane). Using a stereo microscope, individual tetrasporangia could be easily observed on both sides of the translucent thallus. The females were also examined under a stereo microscope for the presence of the emergent cystocarps, which were distinctive because they contained darkly pigmented spores. After these examinations were completed, the plants were rinsed with deionized water, oven-dried in linear polyethylene vials (80°C for four to five days), and weighed at ±0.01 mg.

Experimental Procedure

Range-finding and definitive experiments were performed for each toxicant. Range-finding experiments covered a broad range of concentrations (~10 to 10 000 μg/L). The highest concentration in the final run was selected to cause death or a near-death response. All other concentrations were 60% of the next highest concentration (ASTM Standard Practice E 729-80). Five to eight toxicant concentrations plus a control were used in each definitive run. All the definitive experiments were performed with duplicate flasks at each toxicant concentration. The toxicants were added directly to the culture flasks and were replenished when the media were changed.

Lead nitrate, silver nitrate, cadmium chloride, copper chloride, and potassium cyanide were prepared as concentrated standards in deionized water by Alpha Analytical Laboratories, Jersey City, NJ.[3] The metal solutions were acidified with nitric acid (HNO_3). Only one secondary stock solution was pre-

pared in deionized water from each of these primary stock solutions, to minimize dilution errors. All of the toxicant concentrations were obtained by dispensing from the secondary stock solutions with adjustable micropipets using disposable tips.

The water samples for analysis of toxicant concentrations were taken on Day 0, at each medium change (before the medium was changed), and at the end of an experiment. All heavy metal determinations were made with an atomic absorption instrument (Perkin-Elmer Model No. 360[1]) equipped with a graphite furnace (Perkin-Elmer Model No. 2100[1]). Duplicate 1-mL samples were taken from the control and each dose level at each sampling period. One hundred microlitres of concentrated HNO_3 (ultrapure) was added to each sample prior to analysis. Each sample was compared with two standards in the appropriate concentration range of the sample. The standards for the metal determinations were prepared in seawater of the identical salinity and acidity as the samples. It was required to use D_2 Arc background correction for all of the metal determinations because of the seawater matrix. The day-to-day detection limit of lead, copper, silver, and cadmium were 3, 2, 1, and 0.2 μg/L, respectively. The reproducibility of each elemental determination varied from 100% at their respective detection limits to about 10% at a factor ten times higher than their respective detection limits. Cyanide was determined colorimetrically using a pyridine-barbituric acid reagent after distillation [*11*]. The media from both replicate flasks for each concentration tested were combined at each sampling period, and a single, 500-mL sample was taken for cyanide determination. The analytical data for each toxicant except silver were averaged over the course of the toxicity test. The three lowest concentrations of silver were below the detection limit, therefore only the Day 0 concentrations were used to plot the effects of silver. The Day 0 data for the undetectable concentrations were calculated based on the dilution of the volume of standard added to the flasks.

Data Analysis

The maximum acceptable toxicant concentration (MATC) values were calculated using two different criteria for determining significant differences. The MATCs were determined for vegetative growth of females and tetrasporophytes and for tetrasporangia per milligram dry weight using statistical significance as the criterion for difference. Statistical significance was calculated using analysis of variance followed by Scheffé's multiple-range test (an alpha level of 0.05). The results from the duplicate flasks were pooled for statistical analysis. The MATCs were calculated for cystocarp production and again for production of tetrasporangia using presence or absence to determine the significance. All MATC ranges were determined as the lowest concentration that resulted in a significant difference from the controls and the next lowest concentration [*12*].

Results

Females

Growth (final dry weight) of the females in response to different concentrations of silver, copper, lead, cadmium, and cyanide is shown in Figs. 2, 3, 4, 5, and 6, respectively. The lowest concentration that resulted in a growth response statistically different from that of the control is indicated by an asterisk. The presence of cystocarps (evidence of sexual fusion) is denoted with a "C."

Of all the parameters measured, sexual reproduction (fusion) proved most sensitive to the toxicants used in these experiments. Table 1 lists the MATC ranges for growth of the females and for the presence or absence of cystocarps. Although the MATC values for these two end points are similar, one must keep in mind that the former case dealt with statistically significant decreases, and the latter dealt with complete absence.

Tetrasporophytes

Growth of tetrasporophytes and production of tetrasporangia in response to the various toxicants are shown in Figs. 7, 8, 9, 10, and 11. Statistical significance is indicated by an asterisk. For silver and lead, the MATC values

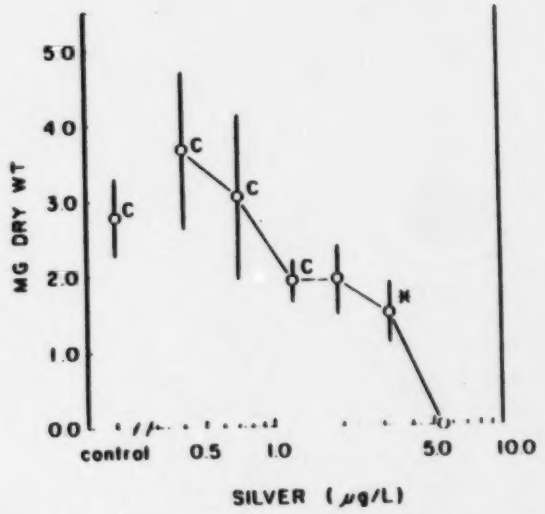

FIG. 2—*Effect of silver on the growth of* C. parvula *female gametophytes. Each point is the mean of ten replicates. Each vertical bar represents one standard deviation unit. The lowest concentration that resulted in a growth response statistically different from that of the control is denoted by an asterisk. The presence of cystocarps (sexual reproduction) is denoted by "C."*

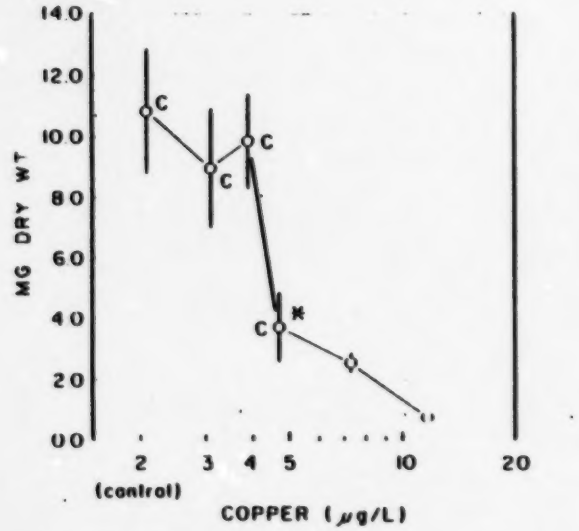

FIG. 3—*Effect of copper on the growth of* C. parvula *female gametophytes. Each point is the mean of ten replicates. Each vertical bar represents one standard deviation unit. The lowest concentration that resulted in a growth response statistically different from that of the control is denoted by an asterisk. The presence of cystocarps (sexual reproduction) is denoted by "C."*

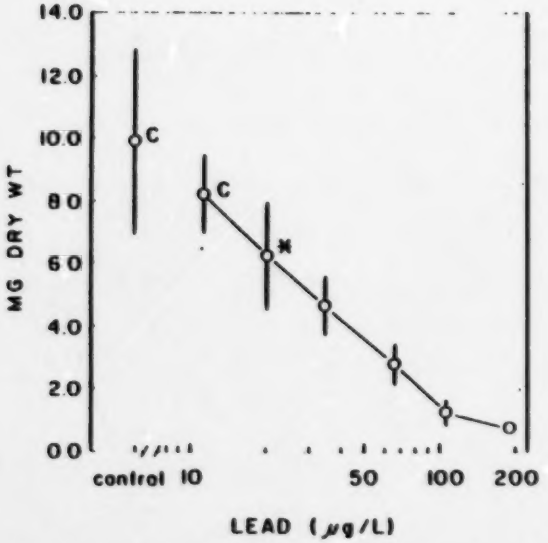

FIG. 4—*Effect of lead on the growth of* C. parvula *female gametophytes. Each point is the mean of ten replicates. Each vertical bar represents one standard deviation unit. The lowest concentration that resulted in a growth response statistically different from that of the control is denoted by an asterisk. The presence of cystocarps (sexual reproduction) is denoted by "C."*

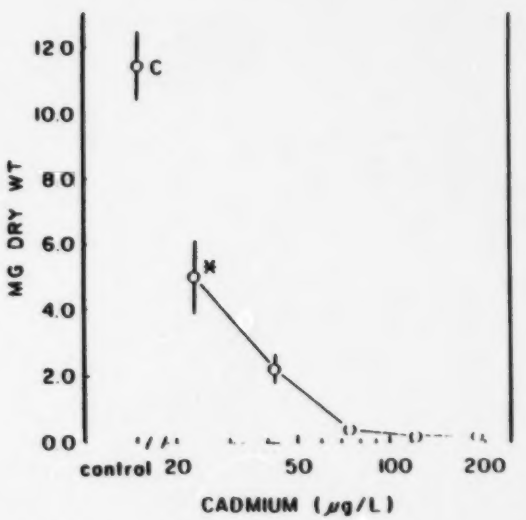

FIG. 5—*Effect of cadmium on the growth of* C. parvula *female gametophytes. Each point is the mean of ten replicates. Each vertical bar represents one standard deviation unit. The lowest concentration that resulted in a growth response statistically different from that of the control is denoted by an asterisk. The presence of cystocarps (sexual reproduction) is denoted by "C."*

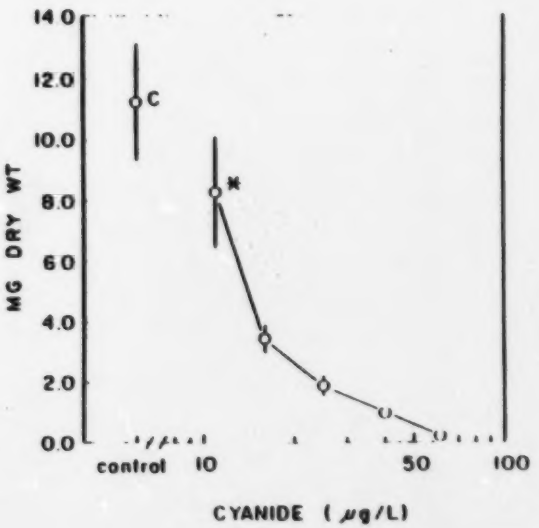

FIG. 6—*Effect of cyanide on the growth of* C. parvula *female gametophytes. Each point is the mean of ten replicates. Each vertical bar represents one standard deviation unit. The lowest concentration that resulted in a growth response statistically different from that of the control is denoted by an asterisk. The presence of cystocarps (sexual reproduction) is denoted by "C."*

TABLE 1—*Maximum acceptable toxicant concentration (MATC) ranges, expressed in micrograms per litre, for static-renewal toxicity tests with female gametophytes of the red alga,* Champia parvula.[a]

	Female Growth, (mg dry weight)	± Cystocarps
Silver	1.9 to 3.2 (2.6)[b]	1.2 to 1.9 (1.6)[b]
Copper	3.9 to 4.7 (4.3)	4.7 to 7.3 (6.0)
Lead	11.3 to 20.3 (15.8)	11.3 to 20.3 (15.8)
Cadmium	0 to 22.8 (11.4)	0 to 22.8 (11.4)
Cyanide	0 to 11 (5.5)	0 to 11 (5.5)

[a]Significant differences were determined by analysis of variance followed by Scheffé's multiple range test for growth of the females and by presence or absence for production of cystocarps.
[b]The numbers in parentheses are means.

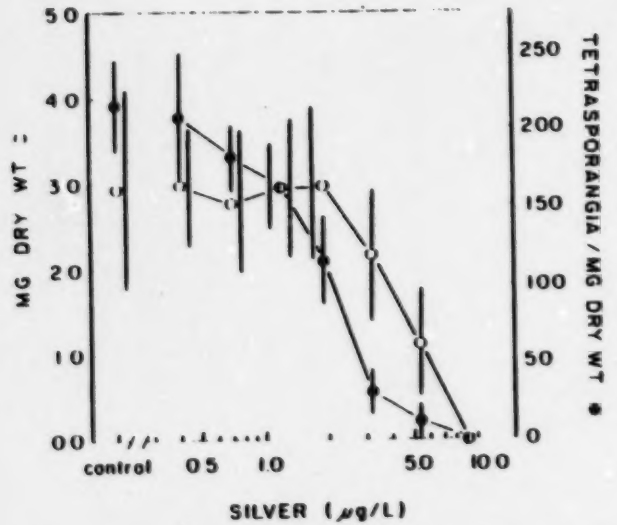

FIG. 7—*Effect of silver on the growth of* C. parvula *tetrasporophytes and production of tetrasporangia. Each point represents the mean of ten replicates. Each vertical bar represents one standard deviation unit. The lowest concentration that resulted in a response statistically different from that of the control is denoted by an asterisk.*

for statistically significant difference for growth (2.6 and 16.2 μg/L, respectively) were equal to those for tetrasporangia per milligram dry weight (Table 2). For the other two heavy metals tested, copper and cadmium, growth of tetrasporophytes was a more sensitive end point (lower MATC) than production of tetrasporangia (Table 2). This was particularly evident with cadmium (Fig. 10). Unlike with the other toxicants, there was no statistically signifi-

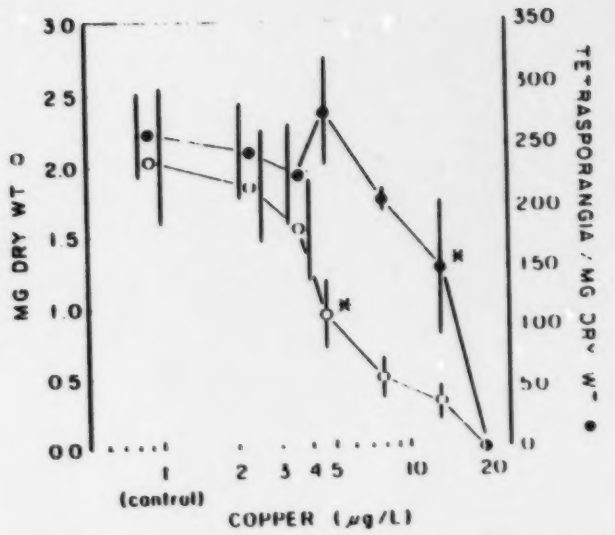

FIG. 8—*Effect of copper on the growth of* C. parvula *tetrasporophytes and production of tetrasporangia. Each point represents the mean of ten replicates. Each vertical bar represents one standard deviation unit. The lowest concentration that resulted in a response statistically different from that of the control is denoted by an asterisk.*

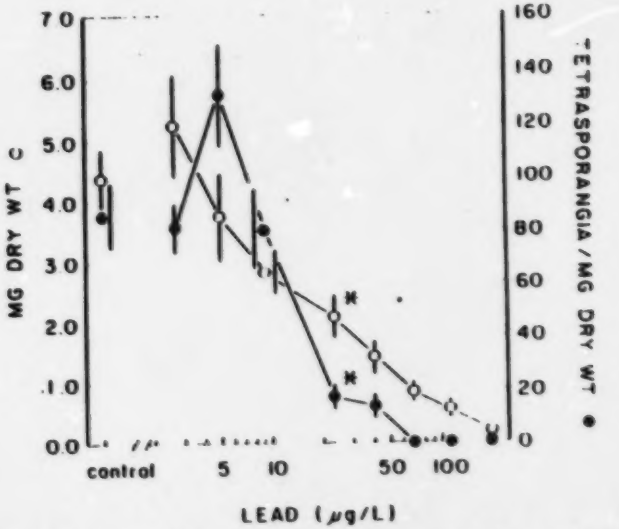

FIG. 9—*Effect of lead on the growth of* C. parvula *tetrasporophytes and production of tetrasporangia. Each point represents the mean of ten replicates. Each vertical bar represents one standard deviation unit. The lowest concentration that resulted in a response statistically different from that of the control is denoted by an asterisk.*

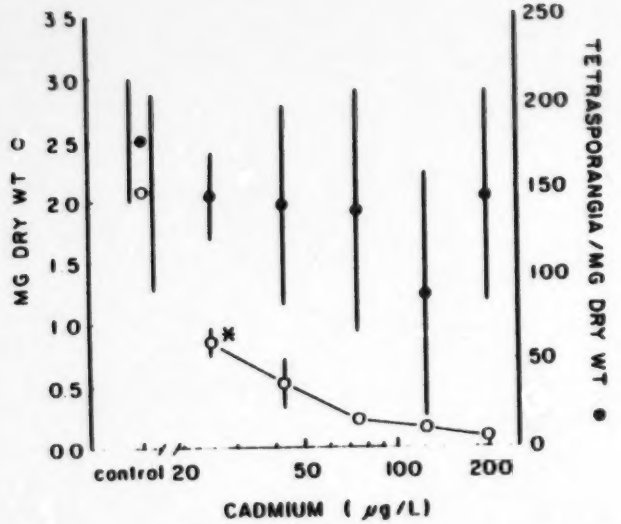

FIG. 10—*Effect of cadmium on the growth of* C. parvula *tetrasporophytes and production of tetrasporangia. Each point represents the mean of ten replicates. Each vertical bar represents one standard deviation unit. The lowest concentration that resulted in a response statistically different from that of the control is denoted by an asterisk.*

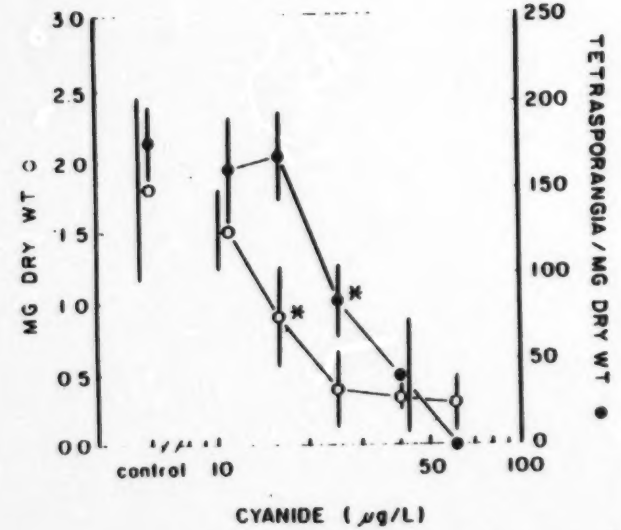

FIG. 11—*Effect of cyanide on the growth of* C. parvula *tetrasporophytes and production of tetrasporangia. Each point represents the mean of ten replicates. Each vertical bar represents one standard deviation unit. The lowest concentration that resulted in a response statistically different from that of the control is denoted by an asterisk.*

TABLE 2—*Maximum acceptable toxicant concentration ranges, expressed in micrograms per litre, for static-renewal toxicity tests with tetrasporophytes of the red alga,* Champia parvula.[a]

	Tetrasporophyte Growth, mg dry weight	Tetrasporangia per mg dry weight	Tetrasporangia
Silver	1.9 to 3.2 (2.6)[b]	1.9 to 3.2 (2.6)	5.4 to 9.0 (7.2)
Copper	3.6 to 4.6 (4.1)	7.8 to 13.3 (10.6)	13.3 to 20.0 (16.7)
Lead	9.1 to 23.3 (16.2)	9.1 to 23.3 (16.2)	40.2 to 68.0 (54.1)
Cadmium	0.0 to 24.9 (12.5)	> 189	> 538[d]
Cyanide	11 to 16 (13.5)	16 to 25 (20.5)	40 to 61 (50.5)

[a]Significant differences were determined by analysis of variance followed by Scheffé's multiple range test for growth of the tetrasporophytes and for production of tetrasporangia. Presence or absence was also used to determine the significant difference for production of tetrasporangia.
[b]The numbers in parentheses are means.
[c]Plants dead at upper limit.
[d]Highest concentration tested.

cant difference between the number of tetrasporangia per milligram dry weight in the control and in any of the cadmium treatments (the highest concentration tested, not shown on the graph, was 538 μg/L). However, the total number of tetrasporangia per plant decreased as the cadmium concentration increased, since this total was directly related to the size of the plants. The large amount of variation in tetrasporangia data for the higher cadmium concentrations reflects the very small dry weights of plants grown at these concentrations. A small difference in the number of tetrasporangia per plant resulted in a very large difference in the number per milligram dry weight.

The response of tetrasporophytes to cyanide (Fig. 11) was similar to that to copper and cadmium in that the MATC range (Table 2) for growth was less than that for tetrasporangia production. Tetrasporangia production ceased at 61 μg/L, but the plants were still alive and growing.

The MATC values for the number of tetrasporangia per milligram dry weight were recalculated using presence or absence as the indicator of significant difference instead of statistical difference. This was done to compare sexual and asexual formation of spores (formation of cystocarps and formation of tetrasporangia, respectively). The MATC values for presence or absence of tetrasporangia ranged from 3 times (for copper) to greater than 47 times (for cadmium) those for cystocarp formation (Tables 1 and 2).

Analytical Data

Table 3 lists the analytical data for a selected concentration of each of the toxicants on Days 0 and 7. The copper, lead, and cadmium concentrations remained relatively stable under the conditions of the experiments (decreasing 5, 6, and 2% respectively). Cyanide decreased about 25% and silver decreased 54%.

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TABLE 3—*Nominal and measured data for a selected concentration of each of the toxicants tested on Day 0 and Day 7, micrograms per litre.*

	Nominal*	Analyzed		Percent Decrease from Day 0 to Day 7
		Day 0	Day 7	
Silver	5.4	5.6	2.6	54
Copper	10.8	13.5	12.8	5
Lead	200	197	186	6
Cadmium	200	202	198	2
Cyanide	125	77	58	25

*Calculated from dilutions of the volume of stock solution added.

Discussion

Although the toxicity test with *C. parvula* is a static-renewal test, the concentrations of cadmium, copper, and lead remained stable under the conditions of the experiments. This presumably is because of the presence of the chelating agent, EDTA, in the medium. Prior growth experiments with *C. parvula* have shown that it will not grow well in static culture in the absence of EDTA. Chelation of iron and other micronutrients is necessary to keep these elements in solution and thus available to the plants. In a static-renewal test, EDTA is apparently necessary to supplement the natural chelates in the seawater. The concentration of EDTA used in these toxicity tests is not the optimum level but is close to the minimum level required for healthy plants. At concentrations below 100 μg/L, plants are stunted and pale yellow instead of the usual reddish brown color.

Work with phytoplankton has shown that copper toxicity is more closely related to cupric ion activity ("free ion") than to total copper concentration [*13*]. However, the present study was not designed to measure free ionic activities of the metals. Current water quality criteria require the measurement of total metal concentration in the water column [*14*]. In addition, measuring free ionic activity is a complicated process since one must take into account the interaction not only of the metal in question with EDTA but also of EDTA with other metals, such as CA^{2+}, Mg^{2+}, and Fe^{3+}. One must also take into account other complexing agents present in the medium, such as CO_2^{2-}, SO_4^{2-}, Cl^-, and other organics present in seawater. Total metal concentration is not only easier to determine than predictions of free ionic activities it is also an environmentally safer parameter, because the chelation capacity of a given body of water would be expected to vary both spatially and temporally. Also, algae are known to excrete organic compounds [*15–17*]. Thus, over the course of a static bioassay, the chelating ability of the culture medium can be expected to change.

An algistatic response has been proposed by Payne and Hall [*18*] as the best end point for toxicity tests with microalgae. This end point would not be useful with macroalgae because asexual and sexual reproduction could be impaired before growth ceases. Tetrasporangia production of *C. parvula* exposed to lead or cyanide was completely inhibited at concentrations of 68 and 61 μg/L, respectively (Figs. 9 and 11), but the plants were still growing at these concentrations. An even better indication was that sexual reproduction ceased long before growth of the female plants stopped (Figs. 2, 3, 4, 5, and 6). Similar effects have been reported for oil in relation to sexual reproduction in the brown algae *Fucus edentatus* and *Laminaria saccharina* and for *C. parvula* [*19,20*]. Pollution assessment with macroalgae must take into consideration reproduction if an accurate picture of the potential harm is to be drawn.

Macroalgae represent a different ecological niche from microalgae. They are generally sessile and also represent a different food source from microalgae, contributing primarily to detrital food chains [*21*]. Currently, the only other algal toxicity tests used routinely employ planktonic microalgae. The level of expertise required to use *C. parvula* is approximately equal to that necessary to run microalgal toxicity tests. The *C. parvula* toxicity test is not intended to replace microalgal tests but rather to complement them by increasing the number of types of plants included in marine toxicity testing.

The toxicity test with *C. parvula* can evaluate simultaneously the effects of compounds on vegetative growth (haploid and diploid life cycle stages) and on reproduction. The test is relatively fast and inexpensive—3 to 4 man hours are needed each week to maintain *C. parvula* stock cultures, and only 10 to 12 are required to complete the testing of any given toxicant. The test requires only a small volume of water. Clonal material is used and is available year round from stock cultures. *C. parvula* is also among the most sensitive organisms for the toxicants tested [*22–26*].

Based on statistical significance, growth of the tetrasporophytes, production of tetrasporangia, and growth of the females generally yielded similar results when determining MATC ranges. The one notable exception was tetrasporangia production in the presence of cadmium. Here it would appear that cadmium had a significant effect on metabolism (that is, growth) whereas it had little effect on meiosis (production of tetraspores). Production of cystocarps yielded MATC values similar to those of the other end points measured (except presence or absence of tetrasporangia). Although all of the end points tested were easy to quantify, sexual reproduction was the simplest because it dealt only with the presence or absence of cystocarps. In addition to being the easiest to measure, production of cystocarps was also the most sensitive of the parameters examined. This is because this parameter dealt with absolute absence. When production of tetrasporangia was examined in a similar manner, the MATC values increased substantially. To make vegetative growth a comparable end point, one would have to use an algistatic response or perhaps death as the criterion for significant difference. This would also

raise the corresponding MATC values. Production of cystocarps as an end point also does not leave any doubt as to whether the observed difference was biologically significant, because absence of cystocarps short circuits the life history. Quantifying sexual reproduction does not require time-consuming counting or drying and weighing, therefore, it has potential as a rapid screening tool to determine the relative toxicities of numerous compounds in a short time period. The results of a rapid screening could then be followed by more extensive algal or animal testing of selected toxicants.

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